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14. ABSTRACT

Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. We have shown that phosphorylation of PR Ser81 is ck2-dependent and progestin-regulated in intact cells. Mutation of the CD domain in PR (mCD PR) abrogates phosphorylation on Ser81, indicating that the CD domain in necessary to facilitate phosphorylation at this site (Ser81). T47D breast cancer cells stably expressing a PR-B mutant that cannot be phosphorylated at Ser81 (S81A) formed fewer soft agar colonies under ligand-independent conditions. Regulation of selected genes by PR-B also required Ser81 phosphorylation for basal and/or progestin-regulated (BIRC3, HSD11β2, and HbEGF) expression. We conclude that phospho-Ser81 PR provides a platform for ck2 recruitment and regulation of selected PR-B target genes. Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Recent clinical data linking the progesterone component of hormone-replacement therapy regimens with the development of breast cancer underscores the importance of understanding how PR works in the context of breast cancer and high kinase environments. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti-cancer agents. Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.

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INTRODUCTION

Progesterone receptors (PR) are critical for massive breast epithelial cell expansion during mammary gland development and contribute to breast cancer progression. Nuclear PR activates transcription of PR-target genes, either directly through binding to progesterone response elements (PREs), or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs). PR is highly post-translationally modified, primarily on N-terminal serine (phosphorylation) and lysine (ubiquitination and sumoylation) residues [1-3]. These modifications significantly alter receptor stability, localization, transcriptional activity and promoter selectivity [4]. In addition to MAPK and cdk2, casein kinase II (ck2), a kinase often overexpressed in breast cancer, has been shown *in vitro* to phosphorylate PR Ser81 [5-7]. Finally, recent clinical data has shown that women taking hormone-replacement therapy whose regimens included estrogen and progesterone, but not estrogen alone, had an increase in breast tumor number and size [8, 9]. In light of these data, understanding how mitogenic protein kinases alter PR is critical to understanding breast tumor etiology and developing better treatments. Progestin-bound PRs induce rapid activation of cytoplasmic protein kinases, leading to regulation of growth-promoting genes by transcription complexes that include phospho-PR species. We propose that hormonal and growth factor signals converge at the level of PR-target gene promoter selection. We identified a putative common docking (CD) domain in the N-terminal B-upstream segment (BUS) of PR-B. [10]. CD domains are regions through which MAPKs (i.e. ERK) interact with their activators, MAPK kinases (MKKs; i.e. MEK1) and inactivators, MAPK-phosphatases (MKPs) [10, 11]. Another nuclear receptor, PPARγ, has also been shown to interact with MEK1 through a similar domain [12]. The PR CD domain, DPSDE, is an exact match to the CD domain of ERK2, suggestive of PR direct binding with MEK1 and/or MKPs. We created a CD domain mutant (mCD PR) that is differentially post-translationally modified following treatment with synthetic progesterone (R5020), as indicated by its lack of phosphorylation-dependent gel retardation, or “up-shift”, when analyzed by Western blotting. These data suggest that mutation of the CD domain disrupts interactions with kinases that are responsible for direct phosphorylation of PR. Because mCD PR fails to up-shift upon ligand-binding, we screened for protein kinases whose target sequences are within close proximity of PR’s CD domain; PR Ser81 is a known ck2 site in the PR N-terminus. ck2 is a ubiquitously expressed, constitutively active kinase that is overexpressed in every cancer examined thus far, including breast cancer [5, 6]. Interestingly, in breast cancer cells treated with highly specific ck2 inhibitors, TBB and DMAT, we observed a loss of the progesterone-dependent PR up-shift, similar to the behavior of the mCD PR mutant. This affect on PR was specific to inhibition of ck2, as treatment with other kinase inhibitors did not affect PR gel mobility following treatment with R5020. These data suggest that ck2 may contribute to protein interactions and/or PR activity via direct phosphorylation of PR. Additionally, these data suggest that protein interactions mediated through the CD domain may affect PR Ser81 phosphorylation. We hypothesize that the PR CD domain mediates direct interactions with mitogenic protein kinases (MEKs, ck2) that phosphorylate PR, thereby dictating downstream signaling and target-gene specificity. In the context of breast cancer where protein kinases are inappropriately activated, hyperactive PR may lead to reprogramming of breast cancer cells, altering their hormone sensitivity and driving breast cancer progression.

BODY

MAJOR RESEARCH TASKS:

***Task 1*: Analysis of the signaling molecules that require the CD domain for PR docking (Months 1-12):**

As reported above, we have created a CD domain mutant PR (mCD PR). To identify possible protein interactions that may be disrupted upon mutation of this domain, we used co-immunoprecipitation (Co-IP) assays to screen for putative interacting proteins. We tested the ability of mCD PR to interact with MKP3, a protein previously shown to interact with ERK2 through an identical CD domain [11]. Using COS cells that had been transiently transfected with wt or mCD PR, as well as myc-tagged MKP3, we showed that while wt PR interacts with MKP3 both in the presence and absence of ligand, mCD PR failed to interact with MKP3 (Fig 1). Co-IP experiments studying a putative interaction between PR and ck2 have thus far been unsuccessful due to limitations in the ability to overexpress ck2. We continue to troubleshoot these experiments, however, the CD domain does not contain sequences known to facilitate interactions between ck2 and its respective substrates, suggesting that a putative interaction between PR and ck2 may be indirect. Co-IPs between PR and other members of the MKP or MEK family have not been tested. These data indicate that PR interacts with MKP3 in a CD domain-dependent manner.

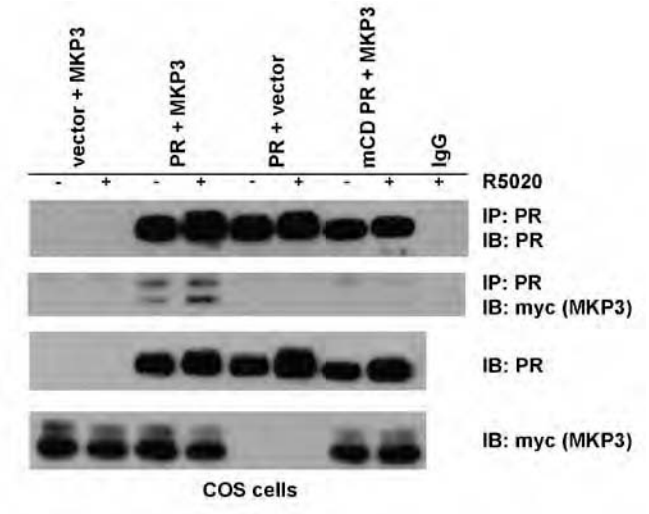

Figure 1. mCD PR fails to interact with MKP3. COS cells were co-transfected with wt or mCD PR, myc-MKP3 or respective vector controls. Following a 24 hr incubation in serum-free media, cells were treated with EtOH or 10nM R5020 for 60 min. Cell lysates were immunoprecipitated with a PR antibody, and the resulting co-immunoprecipitated protein complexes were analyzed by Western blotting (top two panels). Bottom two panels represent total cell lysates.

***Task 2*: Analysis of PR phosphorylation sites that are altered by CD domain interactions (Months 1-12):**

The phosphorylation status of mCD PR in response to ligand was analyzed using phospho-specific PR antibodies. HeLa cells were transiently transfected with wt or mCD PR, and PR phosphorylation in response to ligand was analyzed by Western blotting using antibodies directed to PR Sers 294, 345 and 400 (Fig 2). Interestingly, mCD PR appears to be phosphorylated on an earlier time course as compared to wt PR, with R5020-induced phosphorylation occurring earlier in cells transfected with mCD PR. In contrast, when measuring levels of Ser81 phosphorylation, mCD PR is not phosphorylated on this site in response to ligand (Fig 3). These data suggest that mutation of the CD domain differentially affects PR phosphorylation in a site-specific manner: some sites show hyper-phosphorylation (perhaps due to an altered interaction with a phosphatase, like MKP3 – see Fig 1), whereas other newly characterized PR phosphorylation sites (Ser81; see Appendix A) show decreased phosphorylation in response to ligand, indicating an impaired interaction with a putative PR-modifying kinase, like ck2 (the kinase preliminarily shown *in vitro* to phosphorylation PR on Ser81) [7].

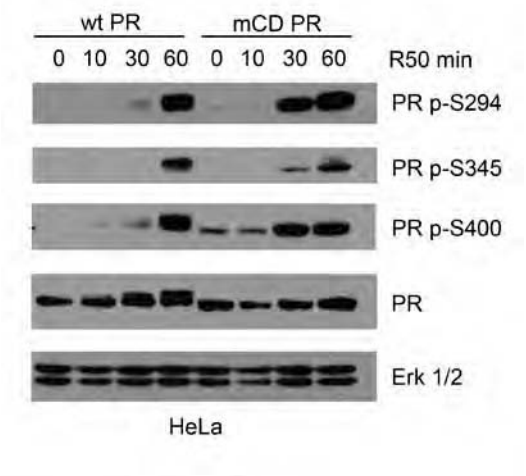

Figure 2. Earlier time-course for progesterone-induced phosphorylation of mCD PR as compared to wt PR. HeLa cells were transfected with either wt or mCD PR. Following transfection, cells were starved for 24 hr in serum-free media and then treated with 10nM R5020 for 0-60 min. Total cell lysates were analyzed by Western blotting.

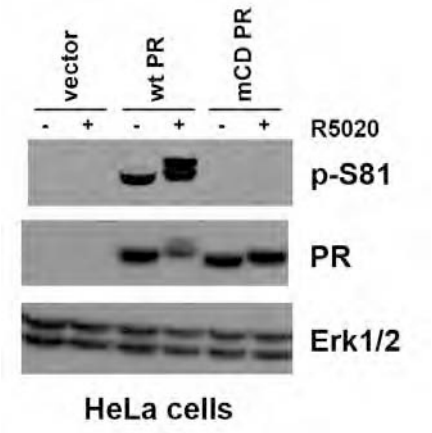

Figure 3. mCD PR lacks phosphorylation on Ser81. HeLa cells were transfected with either wt or mCD PR. Following transfection, cells were starved for 24 hr in serum-free media and then treated with vehicle (EtOH) or 10nM R5020 for 60 min. Total cell lysates were analyzed by Western blotting.

To characterize PR phosphorylation by ck2, the kinase previously shown *in vitro* to phosphorylate PR on Ser81 [7], we analyzed ligand-activated PR phosphorylation in the presence of two highly-specific, synthetic ck2 kinase inhibitors, TBB and DMAT. Data from two different cell lines stably expressing wt PR, HeLa-PR and T47Y-YB, showed that treatment with both inhibitors significantly decreased phosphorylation of Ser81 in response to ligand (Appendix A; Fig 3A-C). We have not yet analyzed the effect of ck2 knockdown (using si/shRNA technology) on Ser81 phosphorylation, but predict that the outcome will be similar to using synthetic kinase inhibitors. These data indicate that PR phosphorylation on Ser81 is regulated by ck2.

***Task 3*: Analysis of CD domain-dependent PR transcriptional activity (Months 6-18):**

Although we have been technically unsuccessful in measuring PR transcriptional activity via PRE-luciferase assays in the presence of ck2 inhibitors (long term inhibition of ck2, as is necessary to measure PR transcriptional products by luciferase, proved to be toxic to both HeLa-PR and T47D-YB cells), we have focused on studying the downstream consequence of ck2 kinase action: phosphorylation on PR Ser81 (thoroughly characterized in Appendix A). To study the functional significance of PR phosphorylation at this site, we created a PR mutant that cannot get phosphorylated by ck2 by mutating Ser81 to alanine (S81A PR). The S81A PR mutant does not get phosphorylated on Ser81, but retains functional transcriptional activity as measured by PRE-luciferase (Appendix A, Fig 4). Stable cell lines were created using this mutant and were used for subsequent experiments (Appendix A, Fig 5). Specifically, T47D-S81A PR cells were used to measure transcription of endogenous PR target genes. We found that Ser81 PR phosphorylation regulated transcription of a subset of PR target genes known to be involved in cell growth and prevention of apoptosis, including BIRC3, HSD11β2 and HbEGF (Appendix A, Fig 6). We have yet to analyze endogenous PR target gene transcription in cells stably expressing mCD PR, but we expect that many of the CD domain-dependent transcriptional targets will overlap with those mediated by S81 phosphorylation, as one primary function of the

CD domain appears to be facilitating phosphorylation at Ser81. Experiments are currently underway to measure endogenous PR gene activity in mCD PR-expressing cells. These data suggest that phosphorylation on Ser81, likely facilitated by the CD domain, regulates a specific subset of PR target gene promoters that regulate cell growth and proliferation genes basally and in response to ligand.

***Task 4*: Analysis of CD domain-dependent rapid signaling events (Months 6-12):**

Experiments to test the ability of mutant PRs (mCD and S81A) to rapidly activate cellular kinases have not yet been initiated.

***Task 5*: Analysis of the effect of PR's CD domain on cell proliferation (Months 12-30):**

Using stable cell lines that express wt, mCD or S81A PR, preliminary experiments were conducted to determine if mutation of the CD domain or phosphorylation on Ser81 affected cell growth in the presence and absence of ligand. Preliminary data obtained from these experiments suggests that cellular proliferation rates are not affected by the aforementioned mutations, as growth rates are similar amongst the cell lines (data not shown; Appendix A). Cell-cycle specific growth analyses have not yet been performed.

***Task 6*: Analysis of the effect of PR's CD domain on anchorage-independent growth (Months 24-36):**

The ability of mCD PR cells to grown in an anchorage-independent manner has not yet been analyzed. However, these experiments have been conducted with regards to S81A PR-expressing cells. Interestingly, cells expressing mutant S81A PR, while retaining their ability to grown soft-agar colonies in response to ligand, formed significantly fewer colonies in the ligand-independent condition as compared to cells expressing wt PR (Appendix A, Fig 5B). These data indicate that phosphorylation on Ser81, in the absence of ligand, contributes to cellular survival as measured by anchorage-independent growth.

KEY RESEARCH ACCOMPLISHMENTS

- Task 1 Milestone: MKP3 was identified as a protein that interacts with PR through the CD domain.
- Task 2 Milestone: Ser81 is differentially phosphorylated due to mutation of the PR CD domain; mCD PR lacks phosphorylation at Ser81. Other PR phosphorylation sites studied appear to be hyper-phosphorylated on mCD PR as compared to wt PR.
- Task 2 Milestone: ck2 is the kinase responsible for phosphorylation of PR on Ser81.
- Task 3 Milestone: A subset of endogenous PR target genes was identified that is regulated by phosphorylation at PR Ser81. This subset contains genes known to regulate cellular proliferation and/or survival.
- Task 5 Milestone: Cellular proliferation rates are likely not affected by mutations in the CD domain or phosphorylation at Ser81.
- Task 6 Milestone: Phosphorylation at Ser81 regulates the ability of PR-expressing cells to survive in an anchorage-independent manner in the absence of ligand.

REPORTABLE OUTCOMES

- Manuscript in review at Molecular and Cellular Biology (Appendix A):

  Hagan, C.R., Regan, T.M., Dressing, G.E. and Lange, C.A. ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B-Isoform-Specific Target Gene Expression in Breast Cancer Cells. ***Mol Cell Biol***, in review.

- Invited presentations (Appendix B):

  Hagan, C.R., Hillard, C.J., Lange, C.A. Signaling Inputs to Progesterone Receptor Action in Breast Cancer Models. FASEB Summer Research Conference: The Physiology of Integrated Nuclear and Extranuclear Steroid Signaling. August 8-13, 2010.

  Hagan, C.R., Hillard, C.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. University of Minnesota Masonic Cancer Center Symposium. June 10, 2010.

- Abstracts presented (Appendix B):
  Hagan, C.R., Hillard, C.J., Faivre, E.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. Jensen Symposium on Nuclear Receptors. October 14-16, 2009.

CONCLUSION

Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. We have shown that phosphorylation of PR Ser81 is ck2-dependent and progestin-regulated in intact cells. Mutation of the CD domain in PR (mCD PR) abrogates phosphorylation on Ser81, indicating that the CD domain in necessary to facilitate phosphorylation at this site (Ser81). T47D breast cancer cells stably expressing a PR-B mutant that cannot be phosphorylated at Ser81 (S81A) formed fewer soft agar colonies under ligand-independent conditions. Regulation of selected genes by PR-B also required Ser81 phosphorylation for basal and/or progestin-regulated (BIRC3, HSD11β2, and HbEGF) expression. We conclude that phospho-Ser81 PR provides a platform for ck2 recruitment and regulation of selected PR-B target genes. Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Recent clinical data linking the progesterone component of hormone-replacement therapy regimens with the development of breast cancer underscores the importance of understanding how PR works in the context of breast cancer and high kinase environments. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti-cancer agents. Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.

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ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B-Isoform-Specific Target Gene Expression in Breast Cancer Cells

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Running title: ck2 regulates Ser81-dependent PR gene expression

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ABSTRACT

Progesterone receptors (PR) are critical mediators of mammary gland development and contribute to breast cancer progression. Progestin-induced rapid activation of cytoplasmic protein kinases leads to selective regulation of growth-promoting genes by phospho-PR species. Herein, we show that phosphorylation of PR Ser81 is ck2-dependent and progestin-regulated in intact cells, but also occurs in the absence of PR ligands, when cells enter the G1/S phase of the cell cycle. T47D breast cancer cells stably expressing a PR-B mutant that cannot be phosphorylated at Ser81 (S81A) formed fewer soft agar colonies. Regulation of selected genes by PR-B, but not PR-A, also required Ser81 phosphorylation for basal and/or progestin-regulated (BIRC3, HSD11β2, and HbEGF) expression. Additionally, wt PR-B, but not S81A mutant PR, was robustly recruited to a PRE-containing transcriptional enhancer region of BIRC3; abundant ck2 also associated with this region in cells expressing wt but not S81A PR. We conclude that phospho-Ser81 PR provides a platform for ck2 recruitment and regulation of selected PR-B target genes. Understanding how PR functions in the context of high kinase activities characteristic of breast cancer is critical to understanding the basis of tumor-specific changes in gene expression and will speed the development of highly selective treatments.

INTRODUCTION

The ovarian steroid hormone progesterone acts by binding to and activating progesterone receptor (PR) A-, B-, and C-isoforms expressed in target tissues. In the normal breast, PR-A and PR-B are typically expressed in a minority population (7-10%) of luminal epithelial cells. PR-B is required for mammary gland development during puberty and pregnancy, and acts by contributing to lobulo-alveolar proliferation and ductal side branching (9, 51). Studies from PR- knockout mice show that these mice have significant defects in mammary gland morphology (primarily PR-B dependent) and reproductive abnormalities (primarily PR-A driven) (51, 53, 59). Additionally, the presence of PR was shown to be required for the formation of mammary tumors in a carcinogen-induced mouse model of breast cancer (52). Finally, recent clinical data has shown that women taking hormone replacement therapy (HRT) whose regimens included both estrogen and a progestin, but not estrogen alone, experienced increased breast tumor number and size (1, 6, 13, 14). Interestingly, the effect of combined HRT on breast cancer risk was reversible (6, 15), suggestive of epigenetic events.

In the absence of progesterone, PR molecules rapidly shuttle between the cytoplasm and the nucleus; cytoplasmic PRs contain membrane-associated species capable of direct binding and signaling to mitogenic protein kinases (c-Src, MAPK, PI3K) (3, 8, 28, 55). Following ligand binding, PRs dissociate from heat shock protein-containing chaperone complexes, undergo dimerization and are largely retained in the nucleus. Nuclear receptors activate transcription of PR-target genes, either directly through

binding to progesterone response elements (PREs), or indirectly through tethering interactions with other transcription factors (AP1, SP1, STATs) (16, 67, 77). Notably, PR is highly post-translationally modified, primarily on serine (phosphorylation) and lysine (acetylation, ubiquitination and sumoylation) residues located in the N-terminal region (19, 20, 49, 85). These modifications are frequently ligand-dependent, but can also occur independently of progestin-binding, and significantly alter receptor stability, localization, tethering interactions, transcriptional activity, and promoter selectivity (84). For example, MAPK and cdk2 have previously been shown to phosphorylate and modulate the activity of both liganded and unliganded PR (45, 49, 68, 88).

The serine-threonine protein kinase ck2 (formerly casein kinase II) is ubiquitously expressed with over 300 substrates, many of which are involved in proliferation, cell survival and gene expression (54). Moreover, ck2 has been shown to be overexpressed in many different types of cancer, including breast cancer (35, 80). ck2 is a unique kinase in that it is constitutively active and does not require modifications or signaling inputs to modulate its kinase activity. In contrast, one mode of ck2 regulation likely occurs via altered subcellular localization of ck2 and/or its respective substrates (30). ck2 localization appears to be altered in a cell-cycle dependant manner, with nuclear accumulation occurring primarily in G1/S (56, 87). However, subcellular sequestration is not the only proposed mechanism for ck2 regulation. Others include regulated assembly of the ck2 holoenzyme, protein complex formation with substrates, autophosphorylation and small molecule interactions (65); little remains known about this topic.

Understanding how a cancer-associated kinase, like ck2, modulates PR function may provide insight into how PR promotes breast cancer cell proliferation and tumor progression (35, 80). ck2 has previously been shown *in vitro* to phosphorylate human PR at Ser81, a residue located in the N-terminal region of PR unique to PR-B, termed the B-upstream segment (BUS) (90). Subsequent *in silico* analysis (i.e. inspection of the PR primary sequence) revealed 11 potential ck2 phosphorylation sites in PR (90). Mass spectrometry studies and *in vitro* kinase assays revealed that Ser81 was the primary site for ck2 phosphorylation; these studies failed to detect phosphorylation on any of the other consensus ck2-sites in PR (90). Herein, we sought to understand the functional significance of ck2 regulation of PR Ser81 in breast cancer models.

MATERIALS AND METHODS

Cell Lines

The estrogen-independent ER/PR positive T47Dco (T47D) variant cell line has been previously described (40). T47D-Y (PR negative), T47D-YB (stably expressing wt PR-B) and T47D-YA (stably expressing wt PR-A) cells were characterized by Sartorius *et al* (73). HeLa-PR cells have been previously described (68). T47D-S81A PR cells were created by stable expression of pSG5-S79/81A PR and pSV-neo in T47D-Y cells using FuGene-HD (Roche). Individual colonies were selected in 500µg/ml G418 and maintained in 200µg/ml G418 after initial selection. The pSG5-S79/81A PR plasmid (containing serine to alanine mutations at Ser79 and Ser81) was generated by GenScript Corporation. T47D-Y and HeLa cells were maintained at 37°C in 5% CO_2 in Minimum Essential Media (MEM; CellGro) supplemented with 5% FBS, 1% Penicillin/Streptomycin, 1% non-essential amino acids, and 6 ng/ml insulin. T47D-YB, T47D-YA, T47D-S81A PR and HeLa-PR cells were maintained under the same conditions, with the addition of 200 µg/ml G418.

Transient transfection experiments were performed as follows: 24hr after cell plating, HeLa cells were transfected with pSG5-vector, pSG5-wt PR or pSG5-S81A PR using FuGene6 (Roche). 24hr following transfection, cells were starved for 18hr in serum-free iMEM (Modified Improved MEM). Following starvation, cells were treated as noted in the respective figure legend and total cell lysates were isolated as described below.

Immunoblotting

For the majority of immunoblotting presented here (exceptions noted in figure legends), cells were starved for 18hr in serum-free iMEM media. Following 18hr starvation, cells were treated, if applicable. Whole cell lysates were isolated using a modified radioimmune precipitation assay (RIPA) buffer (0.15M NaCl, 6mM Na_2HPO_4, 4mM NaH_2PO_4, 2mM EDTA, 1% Triton-X, 0.1M NaF; in H_2O) supplemented with protease and phosphatase inhibitors. Lysates containing equal protein levels (between 25 and 30µg protein was loaded per lane on each gel) were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore) for subsequent immunoblotting analysis. Membranes were probed with primary antibodies recognizing total PR (ThermoScientific #MS-298-P), phospho-Ser294 (Lab Vision Corp. #MS-1332) Erk1/2 (Cell Signaling #9102), and phospho-Erk1/2 (Cell Signaling #9101). The phospho-Ser81 (p-S81) PR antibody was a custom antibody commissioned from Invitrogen designed to recognize the following phospho-specific peptide sequence (PR-B amino acids 76-85): DQQSL-pS-DVEG. Mouse and rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from BioRad, and chemiluminescence was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Company).

Luciferase Transcription Assays

Luciferase assays were performed as previously described (28) using the Dual Luciferase Reporter Assay (Promega). Relative luciferase units (RLU) were normalized to *Renilla* ±SD.

Reagents

Cells were treated with the following reagents (when applicable): R5020 (10nM; Sigma), RU486 (100nM; Sigma), EGF (30 ng/ml; Sigma), TBB (1-100μM; CalBioChem), DMAT (1-100μM; CalBioChem), PP2 (10μM; CalBioChem), Roscovitine (100μM; CalBioChem) and U0126 (10μM; CalBioChem).

Cell cycle analysis/Flow cytometry

1.5 x 10^5 T47D-YB cells were plated in 10-cm^2 dishes in cMEM (day 0). Synchronized cells were treated on day 1 with cMEM containing 2.5μg/mL thymidine (Sigma) for 18hr. Cells were then washed with PBS and fresh iMEM/5% dextran-coated charcoal treated (DCC) serum was added for 7hr. Synchronized cells were then treated for 18hr with iMEM/5% DCC/50μg/mL mimosine. Following the 18hr mimosine treatment, cells were harvested in RIPA for western blotting (as above) or trypsinized and fixed for flow cytometry. For flow cytometry analysis, media and wash (2mL PBS) were collected. Trypsinized cells and collected media/wash were combined, and pelleted by centrifugation. Cells were resuspended in 300μL PBS + 10% FBS, following which 4mL ice cold 80% ethanol was added dropwise to fix samples. Samples were stored at -

20°C until analyzed for cell cycle phase. Fixed cells were pelleted and washed three times with 5mL cold PBS. Samples were resuspended in 100-400µL staining buffer (1XPBS with 10% RNase A (10mg/mL Sigma), 5% FBS, 0.5mM EDTA, 0.1%TX-100, and 200µg/mL propidium iodide (Sigma)). Propidium iodide staining was detected using a FACSCalibur (BD Biosciences). Cells were gated for cell cycle phase using FlowJo (Tree Star Inc.).

Soft Agar Anchorage-Independent Growth Assays

Soft agar assays were performed as previously described (19). Briefly, cells were suspended in 0.48% SeaPlaque GTG Agarose (Lonza) in iMEM supplemented with 5% DCC serum containing either EtOH or 10nM R5020. Cells were plated in triplicate/condition at 9.6 x 10^3/well over a bottom layer of 0.8% agarose/iMEM with 5% DCC serum. Cells were incubated under normal growth conditions for 21 days, following which colonies were counted in 15 fields/treatment group. The data is represented as an average number of colonies per field ± SEM. Soft agar experiments were performed in triplicate.

Real-Time Quantitative PCR (qPCR)

Cells were plated at 5 x 10^5 cells/well in triplicate wells of a 6-well plate. Following 18hr starvation in serum-free iMEM, cells were treated for 1-18hrs with 10nM R5020 or EtOH. Total RNA was isolated using Trizol (Invitrogen); cDNA was created using the

Transcriptor cDNA First-Strand cDNA synthesis kit (Roche) following manufacturer's recommendations. qPCR was performed on equal amounts of cDNA using the Light Cycler 480 SYBR Green1 Master Mix on a Roche 480 Light Cycler. Results in triplicate for each gene of interest were normalized to either β-actin or 18S (as indicated in each respective graph) ± SD.

ChIP assays

ChIP assays were performed using the ChIP-IT Express Kit (Active Motif), according to manufacturer's instructions using sonication as the method for chromatin shearing. Lysates were immunoprecipitated (IP) overnight (18hr) with the following antibodies: PR (ThermoScientific #MS-298-P), ck2α (Santa Cruz sc-12738) or an equal amount of mouse or rabbit IgG. Resulting DNA was analyzed using qPCR as described above, and data is represented as a percentage of input DNA. *In silico* analysis using MatInspector (Genomatix) identified potential PRE-binding sites using the following consensus sequence: RGNACANRNTGTNCY.

Statistics

Statistical significance for all experiments was determined using an unpaired Student's *t* test.

RESULTS

Hormone- and ck2-dependent regulation of PR Ser81 phosphorylation

Previous studies have shown that PR is phosphorylated on Ser81 *in vitro* (90). However, regulation of this site *in vivo* (i.e. in intact cells) has yet to be characterized. Using custom-designed polyclonal antibodies created to recognize PR phospho-Ser81, we measured progestin-induced phosphorylation of this site in T47Dco human breast cancer cells (Fig 1A). T47Dco are unmodified breast cancer cells that naturally constitutively express both PR-A and PR-B, without the requirement of estrogen treatment to induce PR expression (40). We detected weak basal (i.e. in the absence of progestin) PR Ser81 phosphorylation that substantially increased in response to treatment with the synthetic progesterone, R5020 (Fig 1A). Antibody specificity was verified using a Ser81 to alanine PR mutant (S81A), as described below (Fig 4). PR-A does not contain Ser81, located within the BUS domain of PR-B. As expected, our phospho-Ser81-specific antibodies detected no PR-A. In most steroid hormone receptor-positive breast cancer cell models, the levels of PR are primarily upregulated by estradiol, making experimental isolation of PR action (i.e. as studied independently of estrogen) very difficult (38, 39). A naturally occurring PR-negative variant of the T47Dco human breast cancer cell line, termed T47D-Y, was first described by Sartorius and co-workers (73). This parental cell line was used to create stable cell lines constitutively expressing either wild-type (wt) PR-B (T47D-YB) or PR-A (T47D-YA) (73). As observed in unmodified T47Dco cells (Fig 1A), we also detected low basal levels of Ser81 phosphorylation in T47D-YB cells (Fig 1B). Again (as in T47Dco cells), the level of PR

Ser81 phosphorylation increased significantly in response to R5020 (Fig 1B). Control cells not expressing PR (T47D-Y) failed to exhibit any non-specific bands with phospho-S81 or total PR antibodies, indicating a high degree of specificity.

T47D and HeLa cells (stably or transiently expressing PR isoforms) are routinely used as model systems for studying PR action; these cell lines behave similarly with regard to the regulation of post-translational PR modifications and subsequent changes in receptor function (22, 27, 68). To determine the kinetics of PR Ser81 phosphorylation, we analyzed T47D and HeLa cells stably expressing PR-B. Following a timecourse of 10nM R5020 treatment (0min to 6hr), we observed increased Ser81 phosphorylation beginning at 10min (T47D-YB; Fig 1D) to 15min (HeLa-PR; Fig 1C). This reached a maximum level in both cell lines at 30-60min (Figs 1C and D). PR Ser81 phosphorylation preceded the ligand-dependant PR up-shift primarily mediated by phosphorylation events on one or more unidentified residues (79). Note that ligand-dependent downregulation of PR was observed after at least 4hr of R5020 treatment in both cell lines (64).

PR phosphorylation on Ser294, Ser345 and Ser400 occurs in response to either progestins (i.e. R5020) or mitogenic inputs to MAPKs and/or cdk2 (i.e. EGF, serum) (27, 68, 88, 89). To determine the potential for mitogenic inputs to regulate Ser81 phosphorylation, we performed a time course of EGF treatment in HeLa-PR cells (Fig

2A). PR Ser81 phosphorylation was not affected by this mitogen, following up to 60min of EGF treatment, despite significant activation of Erk1/2 over the same timecourse. To test a broader spectrum of mitogens, we used fetal bovine serum (FBS; 20%) as a rich source of multiple growth factors. HeLa-PR cells were grown overnight either in serum-free media, media supplemented with 5% DCC (charcoal-stripped steroid-free media) or full growth media (5% FBS), followed by treatment with either R5020 (positive control for Ser81 phosphorylation; 60min) or 20% FBS (15 or 60min). Only R5020 treatment induced robust PR Ser81 phosphorylation (Fig 2B); no phosphorylation was detected following any of the serum treatments. MAPK (Erk1/2) phosphorylation served as a positive control for serum/mitogenic treatment. Finally, we used the synthetic PR antagonist/partial agonist, RU486, to demonstrate the specificity of PR ligand-induction of Ser81 phosphorylation. HeLa-PR and T47D-YB (Fig 2C) cells were treated with R5020, RU486 or a combination of both. Both ligands induced potent PR Ser81 phosphorylation, while the combination of R5020 plus RU486 was neither additive nor inhibitory. Cumulatively, these data suggest that PR Ser81 phosphorylation occurs primarily in response to progestins, although we frequently observed a low level of basal phosphorylation at this site (see Fig 1; addressed below).

In vitro kinase assays suggest that ck2 directly phosphorylates PR on Ser81 (90). We probed the requirement for ck2 kinase activity in intact cells using two different synthetic, highly specific ck2 kinase inhibitors, TBB and DMAT (26). HeLa-PR and T47D-YB cells were pre-treated with increasing concentrations of either TBB or DMAT

(or DMSO vehicle alone) for 30min, followed by 30min of R5020. Again, PR Ser81 was potently phosphorylated in response to treatment of cells with R5020 alone (30min). However, hormone-induced PR Ser81 phosphorylation was completely blocked with either of the ck2 inhibitors in both HeLa-PR (Fig 3A) and T47D-YB (Fig 3B) cells. We observed a loss of PR protein at high doses of TBB, the more potent of the two ck2 inhibitors. This is likely due to increased PR degradation, as ck2 is a key regulator of the PR chaperone molecule, hsp90; ck2-mediated phosphorylation of hsp90 is essential for its chaperone activity (57, 78). These data suggest that ck2 kinase activity is required for ligand-dependent PR Ser81 phosphorylation. To determine the specificity of this phosphorylation event *in vivo*, we examined Ser81 phosphorylation in the presence of a broad spectrum of inhibitors for kinases known to effect PR phosphorylation at other N-terminal serine residues, including PP2 (c-Src; Ser345), Roscovitine (cdk2; Ser400) and U0126 (MEK1-MAPK; Ser294) (27, 68, 75). HeLa-PR cells were pre-treated with each kinase inhibitor, followed by R5020 for 30min. Again, Ser81 was robustly phosphorylated in response to R5020; this event was completely inhibited only in the presence of ck2 inhibitors (Fig 3C). Together, these data suggest that in the presence of progestin, PR is phosphorylated on Ser81 specifically by (endogenous) ck2.

ck2 has been shown to be regulated in part by cell cycle-dependent localization to the nucleus (56, 87). To further address the potential for ck2-mediated regulation of PR Ser81 in the absence of progestins (i.e. basal phosphorylation levels observed above) we tested the cell cycle dependence of this event. For these studies, T47D-YB cells

were synchronized at the G1/S transition using mimosine, a chemical inhibitor of DNA replication; synchronization of control (vehicle) and mimosine-treated T47D-YB cultures was confirmed by flow cytometry (not shown). In G1/S-synchronized T47D-YB cells, but not vehicle controls, we observed robust PR Ser81 phosphorylation in the complete absence of ligand (Fig 3D), but comparable in magnitude to levels induced following progestin (R5020 or RU486) treatment of unsynchronized cells (Fig 2C). Ser294, a MAPK site primarily regulated only in PR-B, was unaffected by mimosine-induced synchronization (Fig 3D). These data may explain the weak basal phosphorylation of PR Ser81 in unsynchronized cells, indicative of a minority of cells passing through the G1/S phase of the cell cycle, when ck2 is primarily nuclear (56, 87).

PR Ser81-dependent transcriptional activity and promoter selectivity

To investigate the functional consequences of PR Ser81 phosphorylation by ck2, we created Ser81 to Ala phospho-mutant PR. Although nearby Ser79 does not appear to be a PR phosphorylation site, even in the presence of purified ck2 (90), this residue may serve as a phospho-acceptor site when Ser81 is mutated, due to its close proximity. Thus, we mutated both residues (S79/81A, hereby referred to as S81A). Western blotting showed that when transiently transfected into HeLa cells, wt and S81A PR were expressed at equal levels; following treatment with R5020, Ser81 phosphorylation was only detected in cells transfected with wt PR (Fig 4A). Notably, wt and S81A receptors were similarly phosphorylated on all other PR-phosphorylation sites tested (Ser190, Ser294, Ser345 and Ser400; data not shown). To determine if phospho-

mutant S81A PR was capable of binding DNA and subsequently activating transcription, we analyzed wt and mutant PRs using PRE-luciferase reporter gene assays. In transiently transfected HeLa cells treated with vehicle or R5020, wt and S81A PRs behaved similarly (Fig 4B); each receptor activated PRE-luciferase transcription to similar levels (~15-20 fold) in the presence of progestin (Fig 4C). Additional characterization of the S81A PR mutant using confocal microscopy showed no apparent differences in subcellular localization of S81A PR relative to wt PR, both in the presence and absence of ligand (data not shown).

We then created stable cell lines expressing S81A mutant PR in PR-null T47D-Y cells (T47D-S81A). Cells expressing wt PR (T47D-YB) in the same parental cell line background served as controls. Western blotting demonstrated that S81A PR is expressed at similar levels relative to wt PR in this model system (Fig 5A). Again, upon progestin treatment, we detected robust Ser81 phosphorylation in wt, but not S81A, PR-B expressing cells. Additionally, ligand-dependent receptor downregulation, which has been shown to be augmented by MAPK-dependent PR phosphorylation (i.e. at Ser294) (63, 64), followed a similar time course in cell lines expressing either wt or phospho-mutant S81A PR.

In soft agar assays performed *in vitro*, the proliferative and survival effects of progestins are mediated by PR-B, but not PR-A (28). We therefore assayed the ability of S81A

mutant PR to induce breast cancer cell growth in anchorage-independent soft agar assays. Stable T47D cell lines expressing either wt or S81A PR-B, or PR-null cells, were plated for soft-agar colony formation assays in the presence of either vehicle or R5020 (10nM). Following 21 days, established colonies were counted. Cells stably expressing S81A PR retained their ability to form colonies in response to R5020; total numbers of R5020-induced colonies were similar between cells expressing wt or S81A PR by the end of the 21-day assay, while PR-null cells failed to grow well in either condition (Fig 5B). Interestingly, however, cells expressing S81A PR formed significantly fewer colonies in the ligand-independent (basal) condition relative to cells expressing wt PR-B; S81A PR cells resembled PR-null cells in this regard. These data suggest that in the absence of progestin, phospho-Ser81 PR may regulate genes that primarily contribute to cell survival and/or proliferation. Ligand-binding is able to overcome this deficit, perhaps because the same set of genes are also highly responsive to hormone.

Although our PRE-luciferase reporter gene analysis (Fig 4) indicated that S81A PR behaved similarly to wt PR, transcriptional activity on endogenous PR-target genes offers a more sensitive and relevant readout of PR genomic action (i.e. PR-dependent regulation of complex promoters/distant enhancer elements arrayed in chromatin). Additionally, we have shown that PR phosphorylation by rapidly activated cytoplasmic protein kinases provides a mechanism for altered PR-target gene selectivity, recruiting differentially phosphorylated PR species to specific gene subsets (reviewed in (21)).

Using our stable T47D cell line models, we surveyed mRNA expression of several known PR-target genes in the absence and presence of progestin (R5020; 0-18hr) by quantitative real-time PCR (qPCR). While many progestin-regulated genes were similarly expressed in cells containing either wt or S81A PR, others were differentially regulated (see below, Fig 6). These included the previously identified progestin-regulated genes, BIRC3 (70), HSD11β2 (2) and HbEGF (5, 23, 91).

Notably, in the absence of progestin, BIRC3 (Baculovirus Inhibitor of Apoptosis Repeat 3), an anti-apoptosis gene recently identified as a PR-target gene (70), exhibited decreased levels of basal transcription in cells stably expressing S81A mutant PR relative to cells stably expressing wt PR-B (Fig 6A – top). Unliganded PR appears to contribute to basal BIRC3 expression, as PR-null cells (T47D-Y) also contain lower levels of BIRC3 mRNA relative to cells expressing wt PR-B (T47D-YB). Thus, mutation of the Ser81 phosphorylation site in PR appears to have abrogated basal expression of this gene. Additionally, although mutant S81A PR was able to weakly induce BIRC3 mRNA in response to ligand, levels of this transcript never reached those observed in R5020-treated cells containing wt PR-B. Finally, T47D cells stably expressing PR-A (T47D-YA), and thus lacking the BUS region containing Ser81, displayed significantly lower basal expression of BIRC3 and failed to respond to progestin relative to T47D-YB (Fig 6A – bottom), indicating that the structural requirements for regulation of this gene are localized to the segment of PR unique to the B-isoform, which includes the Ser81 phosphorylation site. Together, these data indicate that phosphorylation at PR Ser81

significantly contributes to the basal expression of BIRC3 and is also required for robust responses to ligand.

HSD11β2 (11β-hydroxysteroid dehydrogenase type 2), a cancer-associated proliferative protein (46) that was previously identified as a progestin-responsive gene (2, 24, 83), behaved similarly to BIRC3 in that basal levels of HSD11β2 mRNA were significantly decreased in cells containing mutant S81A PR, as well as in PR-null cells, relative to wt PR-B expressing cells, again strongly suggesting that wt PR Ser81 phosphorylation is responsible for the maintenance of basal transcription of this gene (Fig 6B – top). Similar to the regulation of BIRC3, cells containing S81A PR further enhanced HSD11β2 mRNA expression in response to ligand, while overall transcript levels remained significantly lower relative to those induced in cells expressing wt PR-B. Finally, cells stably expressing PR-A contained similar HSD11β2 mRNA levels to those seen in S81A PR cells (both basally and in response to ligand), again suggesting that regulation of this gene is linked to PR-B-specific phosphorylation of Ser81 (Fig 6B – bottom). These data indicate that PR-B Ser81 phosphorylation primarily regulates the basal expression of these genes (BIRC3, HSD11β2), but can also alter the magnitude of their response to hormone. Taken together with the above effects on soft-agar colony formation (Fig 5B), our data suggest that phospho-Ser81 PR contributes to breast cancer cell survival, even when progestins are absent or limiting.

HbEGF (Heparin-binding epidermal growth factor-like growth factor), is a well-characterized phosphorylation-sensitive PR-target gene shown to be important for growth of mammary epithelial cells (5, 19, 23, 91). In cells expressing wt PR-B, HbEGF mRNA levels were responsive to ligand (Fig 6C – top). In contrast, cells expressing mutant S81A PR failed to induce HbEGF mRNA in response to R5020. Interestingly, in contrast to the previous discussed genes (Figs 6A-B), basal HbEGF transcript levels remained comparable in the absence of ligand in cells expressing either wt PR-A or PR-B, mutant S81A PR or no PR, suggesting that PR does not influence basal transcription of this gene. Cells expressing PR-A and treated with progestin failed to induce HbEGF, again implicating the Ser81-containing region unique to PR-B in the progestin-dependent regulation of this gene (Fig 6C – middle). Finally, cells treated with the ck2 inhibitor, TBB, also failed to induce HbEGF mRNA in response to ligand (Fig 6C – bottom). Together, these data implicate the kinase activity of ck2, presumably through direct phosphorylation of PR Ser81, in progestin-induced upregulation of HbEGF mRNA expression.

Finally, the expression of well characterized PR-target genes including cFos, Tissue Factor (TF) and EGFR (Epidermal Growth Factor Receptor) (43, 61, 62) was not differentially affected either basally or in response to ligand in cells expressing mutant S81A PR as compared to wt PR (data not shown). These genes represent a diverse spectrum of progestin-responsive promoters that display a variety of transcriptional kinetics (i.e. peak mRNA expression) following ligand treatment at 1hr (cFos), 6hr (TF)

and 18hr (EGFR). These data suggest that mutation of the Ser81 phosphorylation site has not disrupted the ability of PR to activate endogenous target genes via general mechanisms (i.e. that may alter all PR transcriptional complexes or effect PR localization), indicating that the genes discussed above are uniquely regulated by phospho-PR Ser81.

Recruitment of phospho-Ser81 PR and ck2 to target gene promoters

To confirm direct regulation of PR-target genes by phospho-Ser81 PR, we performed chromatin immunoprecipitation (ChIP) assays. *In silico* analysis of promoter and enhancer regions of the BIRC3 gene revealed several putative full-length PRE binding sites, including sites located just after the transcriptional start site. ChIP analysis was performed on lysates from EtOH- or R5020-treated cells stably expressing wt or S81A PR, or from PR-null cells, using PR-specific antibodies. In the presence of ligand, we detected robust recruitment (~60-fold) of wt PR to a full-length PRE (PRE1) located within 4kb (downstream) of the BIRC3 transcriptional start site (Fig 7A). This is in contrast to much decreased S81A PR recruitment (~22-fold) to the same area observed in side-by-side assays performed from R5020-treated cells. PR-B recruitment to PRE1 appeared to be highly specific, as other areas tested within the proximal and distal promoter regions were negative for PR binding (data not shown). Interestingly, although we observed significant differences in the basal levels of BIRC3 mRNA expression between cells containing wt and S81A PR (Fig 6), we did not detect appreciable recruitment of PR to PRE1 in the absence of progestin. It is possible that PRE1

primarily regulates the ligand-activated transcriptional response of this gene, whereas another PRE(s) in the region may regulate basal activities and would, therefore, not be detected in our ChIP analyses (focused on PRE1).

To determine if ck2, the kinase responsible for phosphorylation of PR Ser81, and therefore, functional activation of PR-B at Ser81-dependent target genes, was also present at this site, we repeated our ChIP assays using antibodies directed against ck2α, one of the active subunits that comprises the ck2 holoenzyme. Interestingly, ck2α was also strongly recruited to PRE1 in cells containing wt PR-B (~11-fold), but not S81A PR (~2-fold; Fig 7B). These data indicate that in the presence of progestin, both wt PR-B and its activating kinase, ck2, are recruited to PR-binding sites within the transcriptional regulatory regions of BIRC3. Moreover, mutation of PR Ser81 greatly diminished not only PR-B recruitment to this PRE, but recruitment of ck2 as well. We conclude that phospho-Ser81 PR provides a platform for the early recruitment of ck2-containing transcriptional complexes that direct promoter-specific PR-target gene regulation.

DISCUSSION

Our studies reveal novel hormone and cell cycle-dependent regulation of PR Ser81 by ck2, a protein kinase tightly associated with pro-survival and uncontrolled proliferative phenotypes that characterize human malignancy. We show that progestin induces robust ck2-dependent phosphorylation of PR Ser81. Interestingly, this event also occurs in the absence of added PR ligands, during the G1/S transition point of the cell cycle (Fig 3). This result highlights the important linkage that exists between PR and cell cycle regulation (25). Notably, hormone-dependent PR Ser81 phosphorylation is a relatively rapid event, occurring as early as 10min following treatment with PR ligands (R5020 – Fig 1; RU486 – Fig 2). Other potent mitogenic stimuli, including EGF and serum, failed to appreciably induce phosphorylation at this site (Fig 2). Protein kinase inhibitor studies confirmed that ck2 is the kinase primarily responsible for PR Ser81 phosphorylation *in vivo* (Fig 3). Mutational analysis revealed that phospho-mutant S81A PR, while equally transcriptionally active as wt PR in PRE-luciferase reporter gene assays (i.e. a minimal artificial promoter), exhibited dramatically impaired recruitment and transcriptional responses relative to wt PR on selected endogenous PR-target genes (Figs 6-7); PR Ser81 phosphorylation is required for efficient PR and ck2 recruitment to PRE1, located within the BIRC3 downstream enhancer region (Fig 7). Taken together, these data indicate that PR/ck2 complexes may regulate a distinct subset of phospho-Ser81-specific PR-target genes both in the presence and absence of ligand (i.e. in proliferating/cycling cells). Our findings provide novel insight into how PRs may contribute to breast cancer pro-survival and tumor progression, even when hormone concentrations are limiting.

Role of PR phosphorylation events in breast cancer models

Phosphorylation can impact diverse properties of the respective substrate. Direct phosphorylation of PR at specific amino-terminal Ser residues has been shown to alter receptor stability, localization, protein complex formation, dimerization, transcriptional activity and promoter selectivity (reviewed in (84)). Data presented here indicate that tightly regulated (i.e. in response to hormone-binding and/or during G1/S transition) Ser81 phosphorylation directs target gene specificity; we identified at least three PR-target genes that are differentially regulated by phosphorylation at this site. One class of genes is altered both in the presence and absence of progestin (BIRC3 and HSD11β2), while HbEGF is an example of a gene whose expression is primarily ligand- and ck2-dependent (i.e. induced via hormone-regulated PR Ser81 phosphorylation), lacking regulation in the absence of ligand. The precise mechanism(s) through which Ser81 phosphorylation alters target gene specificity is not clear, but might occur via complex mechanisms that may include altered formation of transcriptional complexes and/or recognition/binding affinity for PRE elements and associated regulatory elements, thus altering early events in promoter recruitment (Fig 7 and further discussed below). Related to this finding, phosphorylation on Ser81 contributes in part to PR isoform specificity (Fig 6). The two predominant PR isoforms, PR-B and PR-A, have overlapping but distinct transcriptional profiles (70) and have tissue-specific effects on growth (59, 60), presumably through activation of different subsets of target genes. These receptors are generally expressed at a 1:1 ratio (i.e. equal levels) in normal mammary epithelial

cells, but the ratio of expression is often altered in breast cancers (4, 32, 58). The full-length receptor, PR-B, contains an N-terminal region (the BUS) unique to PR-B where Ser81 is located. Data presented here showing that PR-B-activated gene transcription is lost on selected genes following mutation of the Ser81 phosphorylation site, and that mutant S81A PR-B mimics PR-A in this regard, suggests that Ser81 may be critical for PR-B versus PR-A target gene specificity. Related to this concept, we have begun to explore the possibilities of altered PR-A/B protein-protein interactions with associated transcriptional co-activators, co-repressors and other cofactors. Changes in further post-translational modifications of PR (sumoylation, acetylation, ubiquitination, subsequent multisite phosphorylation events) may also be isoform-specific and dictated in part by early phosphorylation events (19) and/or sequential events (17), but are outside the scope of the present study.

Transcriptional mechanisms are highly ordered and dynamic processes, characterized by waves of interactions between DNA and dozens of regulatory molecules. Given this enormous complexity, the precise role of ck2-dependent PR Ser81 phosphorylation may remain elusive. Notably, preliminary cell fractionation and confocal experiments suggested identical subcellular localization of wt and S81A PR, independent of ligand (data not shown). Additionally, the rate of ligand-dependent downregulation/receptor turnover appeared to be unaltered by Ser81 mutation (Fig 5). Effects on PR dimerization are unlikely, as S81A PR was able to activate PRE-luciferase transcription (Fig 4), as well as regulate other endogenous PR-target genes to levels equal to that of

wt PR (cFos, TF, EGFR). These data indicate that mutant S81A PR is a fully functional transcription factor for some promoters, but not others (i.e. promoter selectivity is primarily altered). Interestingly, less phospho-mutant PR protein appeared to be recruited to a PRE located in the BIRC3 enhancer region relative to wt PR-B (Fig 7). This finding suggests a block at some early event required for efficient PR/DNA recognition and/or interaction. Recent work from Blind *et al* (7*)* suggests that phospho-specific steroid receptor isoforms are differentially recruited to the promoters of specific genes based on their phosphorylation status. Using ChIP analysis, the authors showed that phosphorylation patterns on the glucocorticoid receptor (GR) dictate which gene promoters those phospho-GRs were recruited to, the kinetics of that respective recruitment, and therefore, which GR-target genes were subsequently activated (7). Our data showing decreased recruitment of mutant S81A PR to select PR-target genes (Fig 7) is in concordance with this finding, and suggests that this mechanism of transcriptional regulation may be a characteristic shared by many steroid receptors.

Weak Ser81 phosphorylation occurred in the absence of progestins (Figs 1, 3 and 5) and in cells entering the G1/S boundary (Fig 3D), but was also potently activated in response to progestin. Ligand-binding to PR sets up an exquisite program of cell cycle synchronization (reviewed in (25)). Additionally, PR-target genes include cell cycle mediators and progestin-treated breast cancer cells are known to pause or accumulate at the G1/S boundary (34). Given the tight coupling of PR to cell cycle control, it is perhaps not surprising that selected PR-target genes depend upon Ser81

phosphorylation for regulation both in the presence (HbEGF) and absence (BIRC3, HSD11β2) of ligand. Ligand-independent PR gene regulation may provide important clues to how ck2 is regulated during cell cycle traverse. Protein complex formation involving Ser81-phosphorylated PR and ck2 is the topic of future studies.

Functional significance of ck2 and PR Ser81 target gene regulation in breast cancer

The Ser/Thr protein kinase ck2 is upregulated in every cancer studied thus far (80). Although ck2 itself does not appear to be an oncogene, it is thought that ck2 works in an oncogenic fashion by potentiating the activity of other oncogenes and pro-growth signaling molecules that function as its major substrates (reviewed in (81, 82)). For example, numerous studies have shown that ck2 overexpression promotes tumorigenesis in existing transgenic mouse models of cancer (12, 44, 47, 48). In the context of breast cancer, where progestins have been implicated as a risk factor for tumor development and early progression (1, 6, 14), overexpressed ck2 could further enhance the oncogenic potential of PR through inappropriate phosphorylation (on Ser81). Notably, the genes that are transcriptionally regulated by PR Ser81 phosphorylation have been shown to be important in cell growth, and have each been identified in various types of cancer, including breast cancer. BIRC3 is an anti-apoptosis protein belonging to the Inhibitor of Apoptosis (IAP) family of proteins (18, 70, 71). IAPs bind to and inhibit other pro-death associated proteins, such as caspases, thereby preventing apoptosis (18, 50). BIRC3, a mammalian-specific IAP also known as cellular

IAP2 (cIAP2), is overexpressed, along with other closely related IAP family members, in breast cancer (31, 71, 72). HSD11β2 is a dehydrogenase enzyme that is responsible for the tissue specific metabolism of glucocorticoids (reviewed in (10)). Specifically, HSD11β2 expression has proliferative effects, especially in tumors, through inactivation of the anti-proliferative effects of GR (41, 69). Of note, HSD11β2 is upregulated in many different cancers, including breast, whereas the corresponding normal non-neoplastic tissue normally lacks HSD11β2 expression (41, 46, 83). As a PR-target gene, HSD11β2 may be an important mediator of progestin action. Finally, HbEGF, a gene shown here to be regulated by ligand-induced PR Ser81 phosphorylation, has been shown to contribute to mammary cell proliferation and breast cancer cell growth (5, 23). Moreover, ck2 is frequently upregulated in breast cancer. This fact, coupled with our findings that phospho-Ser81 PR can drive the expression of genes that clearly contribute to breast cancer biology, suggests a scenario for ck2-high breast tumors, in which PR may be inappropriately or persistently phosphorylated on Ser81 (i.e. either basally or in response to ligand) and thereby contribute to a hyperproliferative state. Indeed, we observed increased ligand-independent soft-agar colony formation in cells expressing wt PR-B relative to cells expressing S81A PR and PR-null cells. Thus, the basal level of anchorage-independent growth was abrogated in cells expressing phospho-mutant S81A PR (Fig 5B); cells expressing PR-A also fail to grow in soft-agar (28). Related to this finding, we suspect that many additional pro-survival and/or proliferative genes are regulated by phospho-Ser81 PR. The identification of a more complete Ser81-regulated gene signature awaits detailed gene array analyses.

Due to the diverse nature and subcellular distribution of the >300 substrates of ck2, it is not surprising that ck2 has been localized to nearly every cellular compartment, including, but not limited to, the nucleus, cytoplasm, plasma membrane and mitochondria (reviewed in (29)). Conflicting reports exist regarding a correlation between ck2 localization and cell cycle; this discrepancy is likely due to cell type-specific differences in ck2 distribution. Reports indicate that ck2 localization (either the holoenzyme or specific subunits) shifts to predominantly nuclear during the G1 phase of the cell cycle and at the G1/S border (56, 87). Phosphorylation of PR Ser81 in the absence of ligand (observed in cells arrested at the G1/S transition; Fig 3D) may be regulated as a consequence of increased nuclear accumulation of ck2 observed at this stage of the cell cycle. In addition, extensive work from the Ahmed lab (reviewed in (36)) showed that in response to androgenic or growth factor signals in prostate cancer cells, ck2 localization was strongly nuclear, specifically associating with the nuclear matrix and chromatin, areas of high transcriptional activity (37). It is tempting to speculate that progestins could work similarly to their androgenic counterparts and direct ck2 localization to the nuclear compartment, subsequently activating phosphorylation of downstream substrates, including PR Ser81. Interestingly, PR nuclear entry appears to precede Ser81 phosphorylation (data not shown), similar to the pattern recently described for PR phosphorylation on Ser294 and Ser400 (20). These findings suggest a further link between ck2 localization and Ser81 phosphorylation.

Significantly, nearly 70% of breast cancers express both estrogen receptor-alpha (ER) and PR at the time of diagnosis, in contrast to PR/ER expression in just 7-10% of normal breast luminal epithelium (74). As steroid hormone receptor (SR)-positive tumors progress, they frequently become hormone-independent while retaining receptor expression, indicating an early switch to autocrine or paracrine growth factor signaling (66). In addition, many breast cancers have upregulated protein kinases, such as MAPK, c-Src, cdk2 and ck2, which can modify and hyperactivate PR (33, 76, 80, 86). Recently, progesterone was shown to mediate mammary stem cell self-renewal via paracrine mechanisms in which secreted factors (Wnt, RANKL) derived from PR-positive cells influence the PR-null stem cell niche (42). In PR-positive breast cancer cells, PR action drives proliferation, pro-survival signaling, and early invasion primarily by autocrine mechanisms (11, 28, 67). In an environment where steroid hormones are no longer required to drive cellular proliferation (i.e. during SR-positive tumor progression), the increased expression and constitutive activation of PR-activating protein kinases may promote increased cell survival and uncontrolled growth (i.e. in the face of endocrine therapies primarily directed against ER). Understanding how mitogenic protein kinases, such as ck2, alter PR phosphorylation and function is critical to fully understanding breast tumor etiology and developing better targeted therapies. Due to the ubiquitous nature of ck2 and its prevalence in many different types of cancer, there has been much interest in the development of ck2 inhibitors as anti-cancer agents (81). Clinical ck2 inhibitors, in combination with more specific anti-progestins (new classes of selective progesterone receptor modulators or SPRMs), could provide an effective combination of targeted therapy for breast cancer treatment.

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Figure1

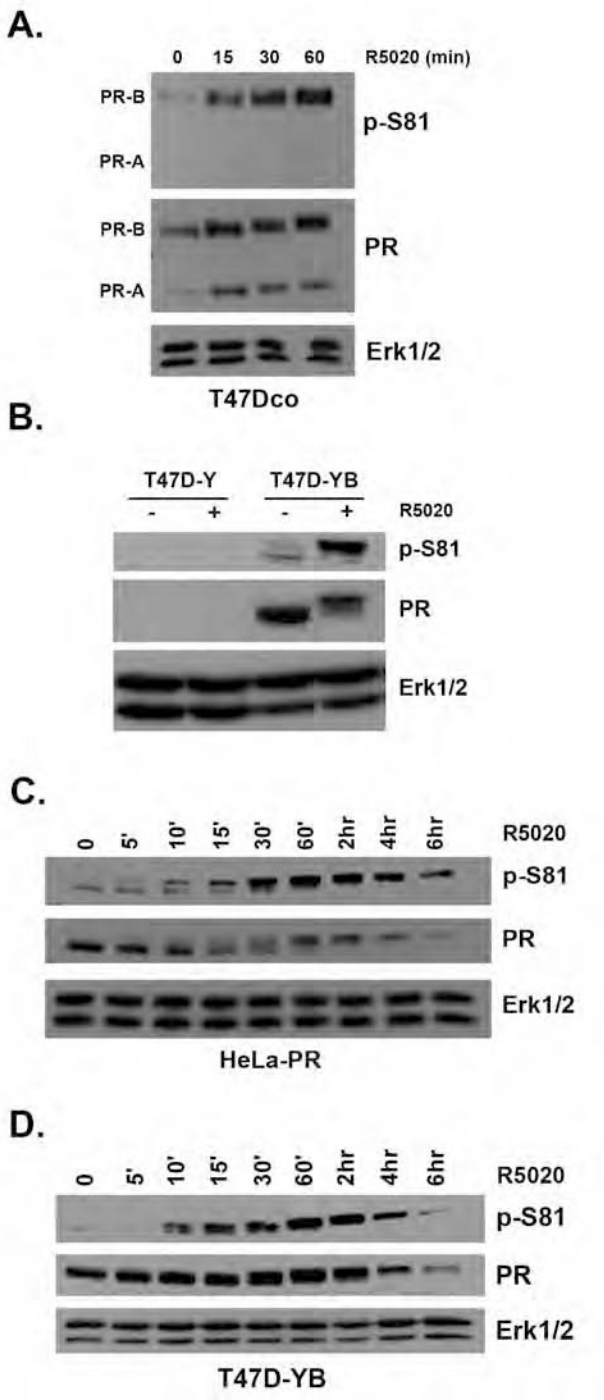
A.
0 15 30 60 R5020 (min)
PR-B
PR-A
p-S81
PR-B
PR-A
PR
Erk1/2
T47Dco
B.
T47D-Y
T47D-YB
- + - +
R5020
p-S81
PR
Erk1/2
C.
0 5' 10' 15' 30' 60' 2hr 4hr 6hr
R5020
p-S81
PR
Erk1/2
HeLa-PR
D.
0 5' 10' 15' 30' 60' 2hr 4hr 6hr
R5020
p-S81
PR
Erk1/2
T47D-YB

Figure 1. *In vivo* phosphorylation of PR Ser81.

A. T47Dco cells were starved for 18hr in serum-free media followed by treatment with 10nM R5020 or ethanol (vehicle) for 0-60min. Lysates were analyzed by Western blotting using antibodies against total Erk1/2 (loading control), total PR and a custom-designed antibody that specifically recognizes phosphorylated Ser81 PR (p-S81). B. Cells lacking PR (T47D-Y) and cells stably expressing PR-B (T47D-YB) were serum-starved for 18hr and then treated with 10nM R5020 or EtOH for 60min. Lysates were analyzed by Western blotting as described in A. C and D. Following 18hr serum starvation, HeLa cells (C) stably expressing wt PR (HeLa-PR) and T47D-YB (D) cells were treated with a time course of 10nM R5020 for 0min-6hr. Lysates were analyzed by Western blotting as described in A.

Figure 2

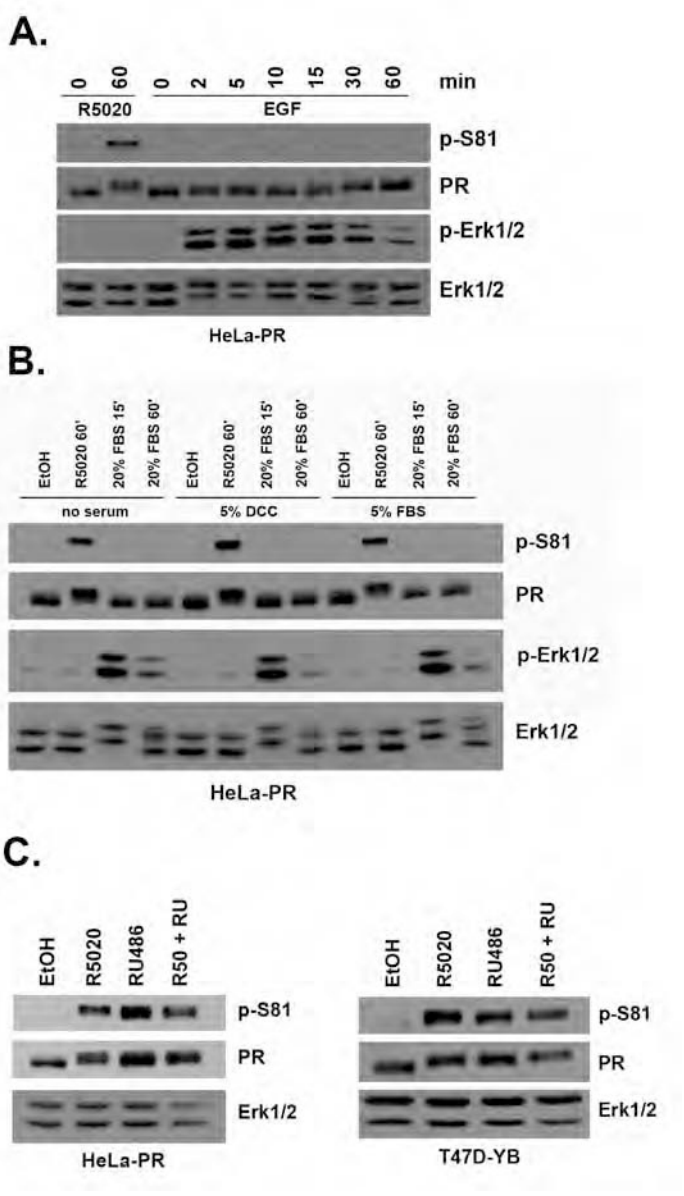

Figure 2. Ligand-dependent PR Ser81 phosphorylation.

A. HeLa cells stably expressing PR (HeLa-PR) were starved for 18hr in serum-free media, then treated with 10nM R5020 for 0-60min, a time course of EGF (30 ng/ml) for 0-60min, or vehicle controls. Lysates were analyzed by Western blotting using p-S81,

PR, p-Erk1/2 (control for EGF treatment) or total Erk1/2 antibodies. B. HeLa-PR cells were starved in media containing no serum, 5% charcoal-stripped steroid-free media (5% DCC), or 5% Fetal Bovine Serum (FBS) for 18hr. Cells were then treated with 10nM R5020 for 60min, 20% FBS for 15 or 60min, or vehicle control (EtOH). Lysates were analyzed by Western blotting as described in A. C. Following 18hr serum starvation, HeLa-PR or T47D-YB cells were treated with 10nM R5020 or 100nM RU486, both or vehicle control (EtOH). Lysates were analyzed by Western blotting as described in A.

Figure 3

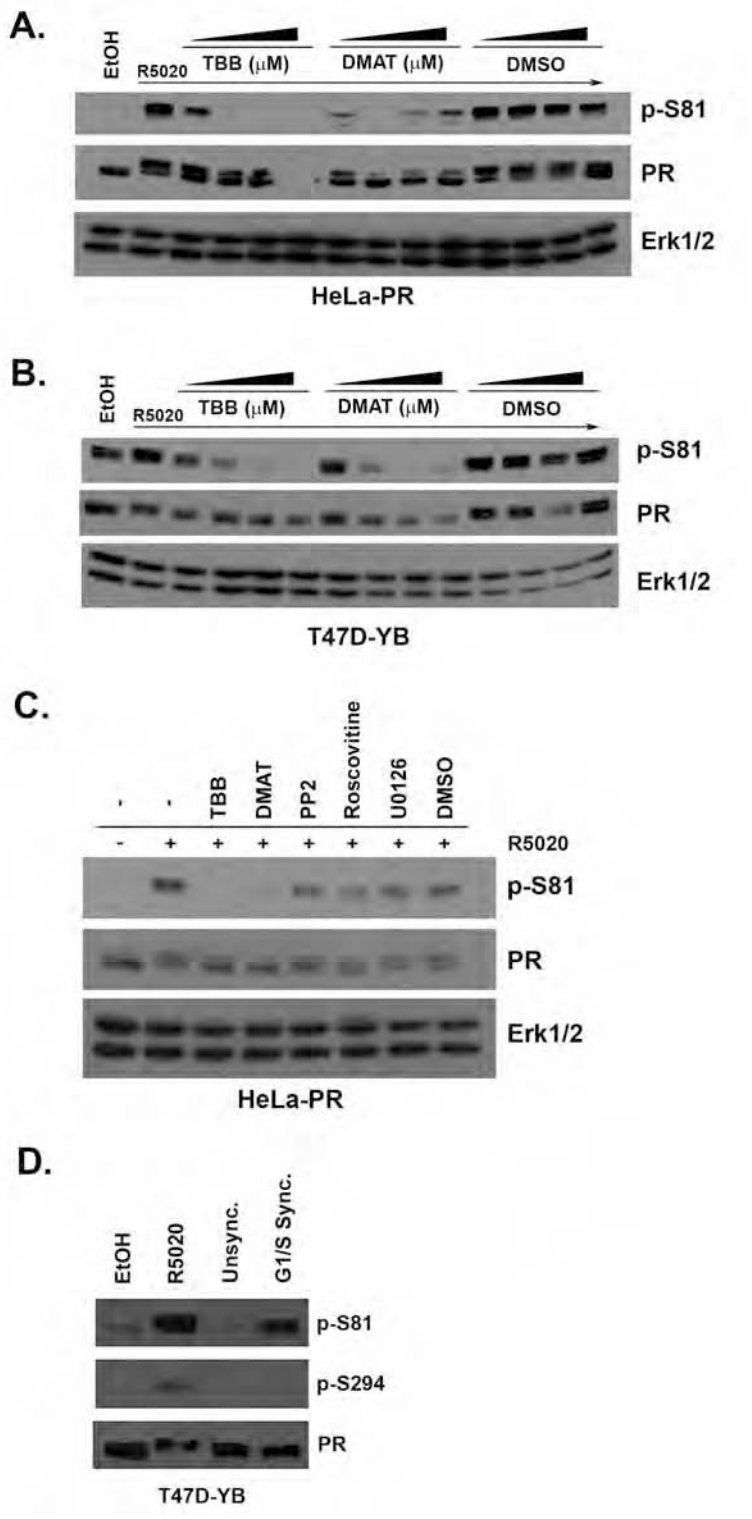
A.
EtOH
R5020
TBB (μM)
DMAT (μM)
DMSO
p-S81
PR
Erk1/2
HeLa-PR
B.
EtOH
R5020
TBB (μM)
DMAT (μM)
DMSO
p-S81
PR
Erk1/2
T47D-YB
C.
-
-
TBB
DMAT
PP2
Roscovitine
U0126
DMSO
-
+
+
+
+
+
+
+
R5020
p-S81
PR
Erk1/2
HeLa-PR
D.
EtOH
R5020
Unsync.
G1/S Sync.
p-S81
p-S294
PR
T47D-YB

Figure 3. PR Ser81 is phosphorylated by ck2.

A and B. HeLa-PR (A) and T47D-YB (B) cells were serum-starved for 18hr. Cells were then pre-treated with increasing doses of TBB (1-100μM), DMAT (1-100μM) or DMSO (vehicle) for 30min, followed by 10nM R5020 for 30min. Alternatively, cells were treated with R5020 for 30min or vehicle (EtOH) with no pretreatment. Lysates were analyzed by Western blotting using p-S81, PR and Erk1/2 antibodies. C. HeLa-PR cells were starved for 18hr in serum-free media. Cells were then pre-treated (30min) with TBB (10μM), DMAT (10μM), PP2 (10μM), Roscovitine (100μM), U0126 (10μM), vehicle (DMSO) or left untreated. Following kinase inhibitor pre-treatments, cells were treated with 10nM R5020 or vehicle (EtOH) for 30min. Lysates were analyzed by Western blotting as described in A. D. T47D-YB cells were serum-starved for 18hr and treated with EtOH or 10nM R5020 for 60min (left two lanes). Alternatively, cells were treated sequentially as follows: 18hr with thymidine (2.5μg/ml) or vehicle (PBS), iMEM plus 5% DCC for 7hr, iMEM/5% DCC/mimosine (50μg/ml; G1/S Sync.) or vehicle (Ammonium hydroxide; Unsync.) for 18hr. Following synchronization (confirmed by flow cytometry; not shown), protein was analyzed via Western blotting with antibodies for p-S81, phospho-Ser294 (p-S294) or PR.

Figure 4

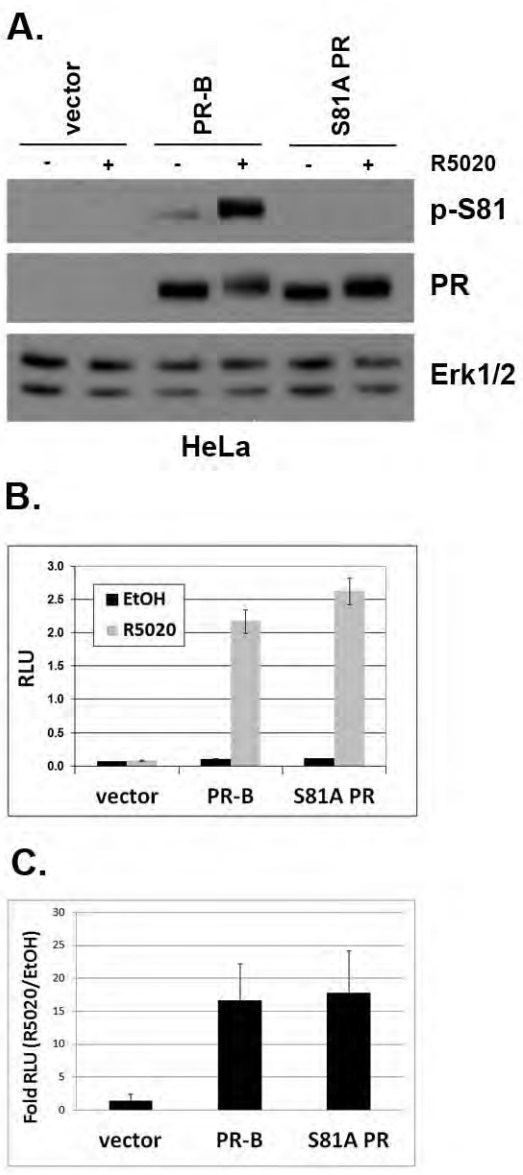

Figure 4. S81A PR phospho-mutant is transcriptionally active.

A. HeLa cells were transiently transfected with wt PR-B, S81A PR or empty vector alone. 24hr following transfection, cells were starved for 18hr in serum-free media and then treated with 10nM R5020 for 60min. Lysates were analyzed via Western blotting

using p-S81, PR and Erk1/2 antibodies. B and C. HeLa cells were transiently transfected with plasmids expressing wt PR-B, S81A PR or vector only, as well as a firefly PRE-luciferase reporter construct and *Renilla* expression control. 24hr following transfection, cells were starved for 18hr in serum-free media, followed by an 18hr 10nM R5020 treatment. Relative luciferase units (RLU) are plotted as a function of firefly PRE-luciferase over *Renilla* luciferase controls. Error bars are ±SD of triplicate measurements (B). C. Fold RLU of R5020-treated cells over EtOH-treated cells. Error bars represent the ±SD of three independent experiments.

Figure 5

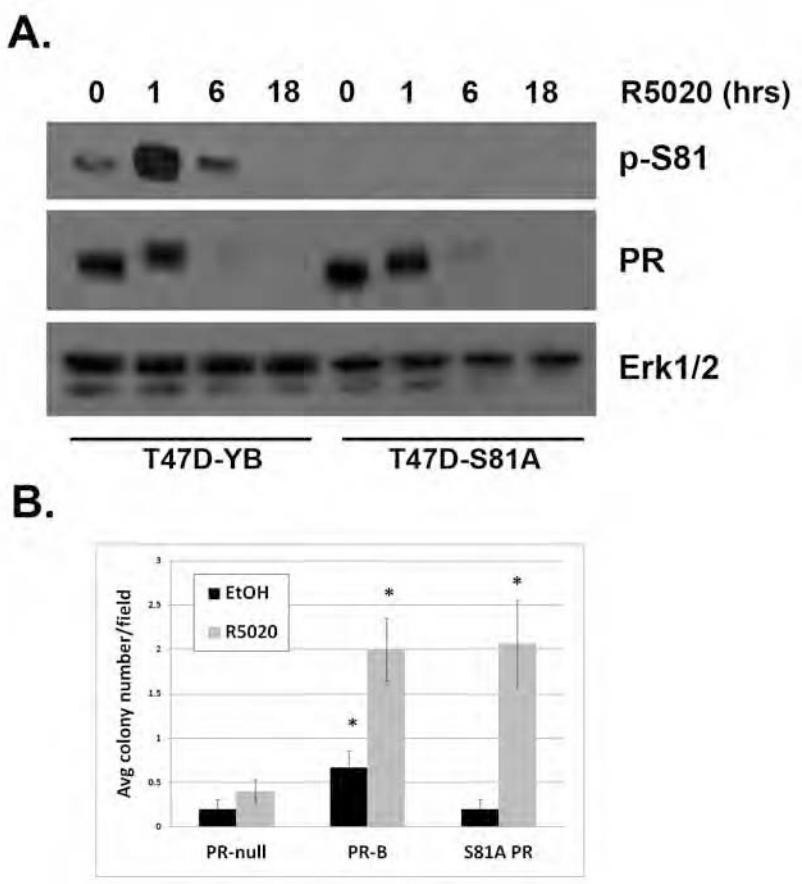

Figure 5. Stable S81A PR cell lines have impaired anchorage-independent survival in soft agar.

A. T47D-Y cells stably expressing wt PR-B (T47D-YB) or S81A PR (T47D-S81A) were serum-starved for 18hr, and then treated with 10nM R5020 for 0-18hr or vehicle (EtOH; 18hr). Lysates were analyzed by Western blotting using p-S81, PR and Erk1/2 antibodies. B. T47D-Y cells (PR-null) or T47D cells stably expressing PR-B or S81A PR were plated in soft agar containing 5% DCC media, and either EtOH or 10nM R5020 for

21 days. Colonies were counted in 15 fields/treatment group and error bars represent the standard error of the mean (SEM) of these measurements. Soft agar assays were performed in triplicate with similar results. Asterisks (*) indicate statistical significance ($p<0.05$; determined using an unpaired Student's *t* test) as compared to the respective treatment group (EtOH or R5020) in control cells (PR-null).

Figure 6

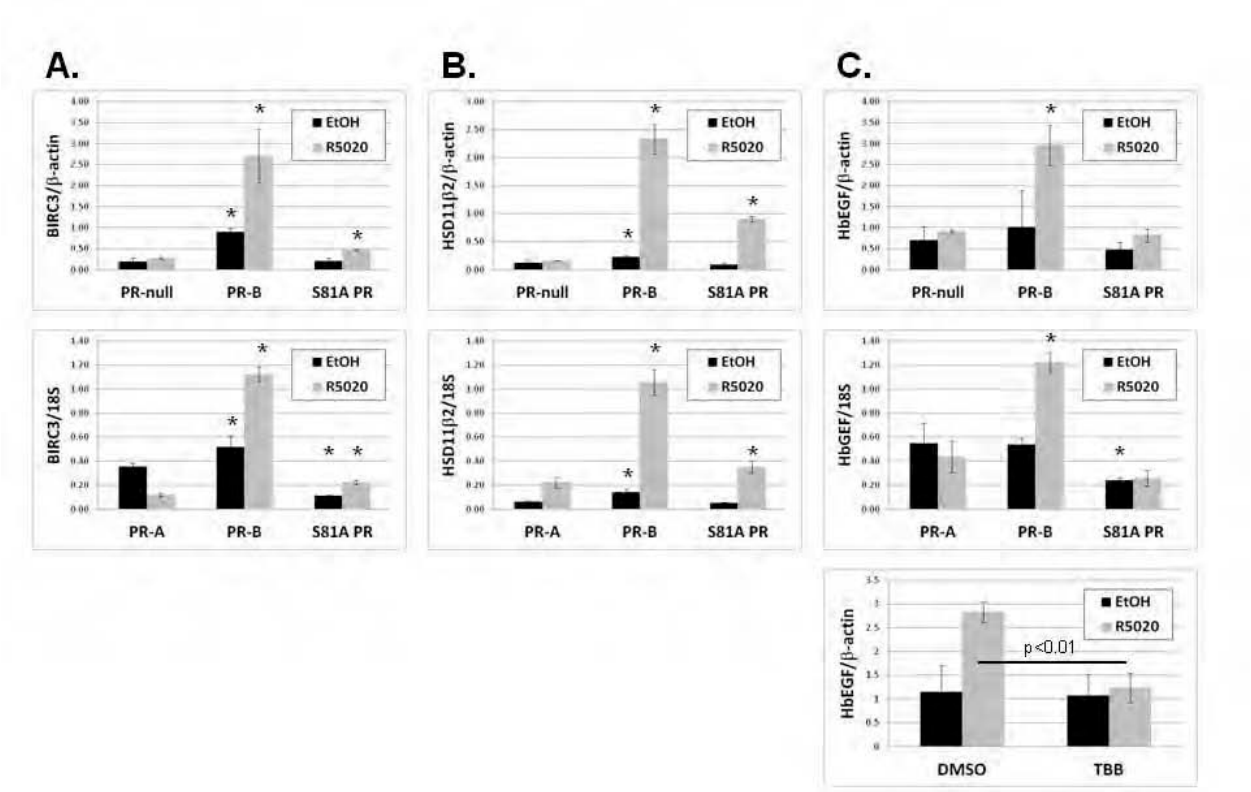

Figure 6. Endogenous PR-target gene expression is attenuated in cells containing S81A PR relative to wt PR.

A, B, and C. Top: T47D-Y cells stably expressing either wt PR-B or S81A PR, or unmodified (PR-null) cells, were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 or EtOH for 6hr. BIRC3 (A), HSD11β2 (B), HbEGF (C), or β-actin (internal control) mRNA levels were analyzed by qPCR. Middle: T47D-Y cells stably expressing wt PR-A, PR-B or S81A PR were serum-starved for 18hr, followed by

treatment with 10nM R5020 or EtOH for 6hr. BIRC3 (A), HSD11β2 (B), HbEGF (C), or 18S (internal control) mRNA levels were analyzed by qPCR. Asterisks (*) indicate statistical significance ($p<0.05$; determined using an unpaired Student's *t* test) as compared to the respective treatment group (EtOH or R5020) in control cells (PR-null or PR-A). Bottom (C): T47D-YB cells were starved for 18hr in serum-free media. Cells were then pretreated with TBB (10μM) or DMSO (vehicle) for 30min, followed by 60min of 10nM R5020. HbEGF and β-actin (internal control) mRNA expression was analyzed using qPCR. Error bars represent ±SD of triplicate measurements.

Figure 7

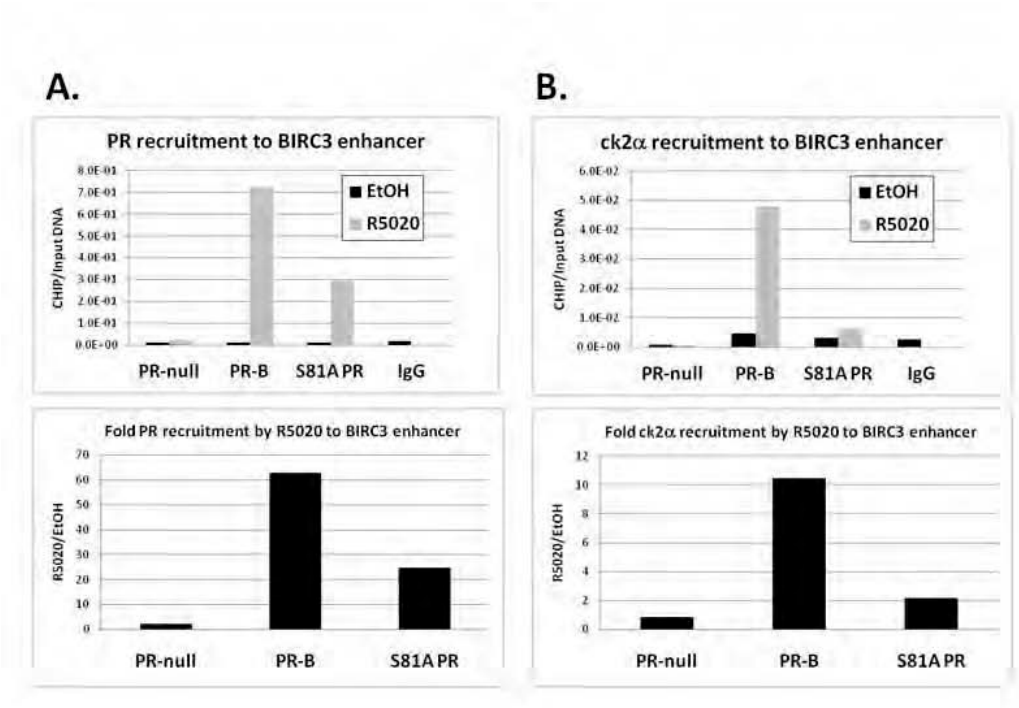

Figure 7. Decreased recruitment of S81A PR and ck2α to a PRE-containing BIRC3 enhancer region.

A and B. Top: T47D-Y cells stably expressing either wt PR-B or S81A PR or unmodified cells (PR-null) were serum-starved for 18hr. Cells were then treated with EtOH or 10nM R5020 for 60min. Fixed lysates were subjected to ChIP with antibodies against PR-B (A) or ck2α (B), and qPCR was performed on the isolated DNA using primers designed to amplify a PRE-containing enhancer region of BIRC3 (+3377 to

+3391). Species-specific IgG antibodies were used as controls (IgG). Bottom: Fold recruitment of PR or ck2α in R5020 condition over EtOH. ChIP experiments were performed in triplicate and a representative experiment is shown.

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carcinoma in situ correlates with high tumour grade, high proliferation and HER2 positivity. Br J Cancer **95:**1410-4.

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90. **Zhang, Y., C. A. Beck, A. Poletti, D. P. Edwards, and N. L. Weigel.** 1994. Identification of phosphorylation sites unique to the B form of human progesterone receptor. In vitro phosphorylation by casein kinase II. J Biol Chem **269:**31034-40.

91. **Zhang, Z., C. Funk, D. Roy, S. Glasser, and J. Mulholland.** 1994. Heparin-binding epidermal growth factor-like growth factor is differentially regulated by

progesterone and estradiol in rat uterine epithelial and stromal cells. Endocrinology **134:**1089-94.

CURRICULUM VITAE

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EDUCATION

2000-2006 University of Chicago, Chicago, IL
Committee on Cancer Biology
Mentor: Charles Rudin, MD, PhD
Degree granted: Ph.D. in Cancer Biology
Dissertation: *Induction of mobile genetic elements following exposure to DNA-damaging agents*

1994-1998 Colorado College, Colorado Springs, CO
Degree granted: B.A. in Biochemistry, *cum laude*

RESEARCH EXPERIENCE

2008 to current Postdoctoral Fellowship, University of Minnesota
Mentor: Carol Lange, PhD
Research Focus: Studying the regulation of the progesterone receptor by MAPKs.

2006 to 2008 Postdoctoral Fellowship, Northwestern University
Mentor: Vincent Cryns, MD
Research Focus: Studying role of αB-crystallin in protecting breast cancer cells from chemotherapy-induced apoptosis.

2000 to 2006 Graduate Student, University of Chicago, Chicago, IL
Mentor: Charles Rudin, MD, PhD
Research Focus: Defining pathways responsible for activating movement of retrotransposons in response to genotoxic stress. Research also entails investigating how retrotransposition of these elements may be related to the etiology of secondary malignancies.

1998-2000 Pre-Doctoral Fellow, Human Gene Therapy Research Institute,
Des Moines, IA
Mentor: Charles Link, MD, PhD

Research Focus: Concentrating radioisotope in cancer cells using the sodium/iodide symporter gene as a novel approach to cancer gene therapy. Secondary project involved restoring DNA repair activity in xeroderma pigmentosum cells using the cloned T4 endonuclease V gene.

1997-1998 Organic Chemistry Lab Assistant, Colorado College,
Colorado Springs, CO
Primary Duties: Instructed students in basic organic chemistry lab techniques, such as NMR, IR, TLC and MS; responsible for student safety in the lab; graded lab related assignments, including lab notebooks, tests, and lab technical competency tests.

HONORS, AWARDS AND PROFESSIONAL MEMBERSHIPS

Honors and Awards

Brigid G. Leventhal Women in Cancer Research Scholar, awarded for the presentation of a meritorious scientific paper, 96th Annual Meeting of the American Association for Cancer Research, 2005

University of Chicago Doolittle Fellowship, awarded to students with outstanding academic achievement for travel to a scientific meeting, 2005

University of Chicago Women's Board Travel Fellowship, awarded to students presenting a meritorious abstract at a scientific meeting, 2005

University of Chicago Doolittle Fellowship, awarded to students with outstanding academic achievement for travel to a scientific meeting, 2003

Aventis Scholar in Training, awarded to students and fellows whose proffered papers are highly rated by the judging committee, 93rd Annual Meeting of the American Association for Cancer Research, 2002

Cum laude, B.A. in Biochemistry, Colorado College, 1998

Alpha Lambda Delta Honor Society, Colorado College, 1998

Outstanding Achievement in Biochemistry Award, awarded to the graduating student with the strongest academic record, Colorado College, 1998

Dean's List, Colorado College, 1994-1998

Grants/Funding

DOD Breast Cancer Research Program Post-doctoral Award; 2009-2012

Komen Grants Program, Post-doctoral Fellowship Award; awarded 2009-2012, declined due to acceptance of DOD award

American Cancer Society, Post-doctoral Fellowship; awarded for 2009-2011, declined due to acceptance of DOD award

Komen Grants Program, Post-doctoral Fellowship Award; awarded 2008, declined due to institutional relocation

Institutional National Research Service Award (University of Minnesota), National Cancer Institute, awarded for postdoctoral fellowship salary support, 2008-2009

Institutional National Research Service Award (Northwestern University), National Cancer Institute, awarded for postdoctoral fellowship salary support, 2007-2008

Penny Severns Post-doctoral Fellowship, Illinois Department of Public Health; awarded for 2007 fiscal year

Institutional National Research Service Award (Northwestern University), National Cancer Institute, awarded for postdoctoral fellowship salary support, 2006-2007

Professional Memberships

Member, Women in Endocrinology, 2008-present

Member, The Endocrine Society, 2008-present

Member, Women in Cancer Research, 2003-present

Associate Member, American Association for Cancer Research, 2002-present

Member, American Association for the Advancement of Science, 1997-present

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Publications

Hagan, C.R., Regan, T.M., Dressing, G.E. and Lange, C.A. ck2-Dependent Phosphorylation of Progesterone Receptors (PR) on Ser81 Regulates PR-B-Isoform-Specific Target Gene Expression in Breast Cancer Cells. ***Mol Cell Biol***, in review.

Hagan, C.R., Faivre, E.J., and Lange, C.A. Scaffolding Actions of Membrane-Associated Progesterone Receptors. ***Steroids*** 2009 Jul;74(7):568-72.

Dressing GE, **Hagan CR**, Knutson TP, Daniel AR, Lange CA. Progesterone receptors act as sensors for mitogenic protein kinases in breast cancer models. ***Endocrine-Related Cancer*** 2009 Jun;16(2):351-61.

Carbajal L, Deng J, Dressing GE, **Hagan CR**, Lange CA, Hammes SR. Meeting review: Extra-nuclear steroid receptors-Integration with multiple signaling pathways. ***Steroids*** 2009 Jul;74(7):551-4.

Hagan, C.R. and Rudin, C.M. DNA cleavage and Trp53 differentially effect SINE transcription. ***Genes Chromosomes and Cancer*** 2007 Mar;46(3):248-60.

Hagan, C.R., Sheffield R.F., and Rudin, C.M. Human Alu element retrotransposition induced by genotoxic stress. ***Nature Genetics*** 2003 Nov;35(3): 219-20.

Heltemes, L.M., **Hagan, C.R.**, Mitrofanova, E.E., Panchal, R.G., Guo, J. and Link, C.J. The sodium iodide symporter gene permits more effective radioisotope concentration than the human sodium iodide symporter gene in human and rodent cells. ***Cancer Gene Therapy*** 2003 10(1): 14-22.

Mitrofanova E.**, Hagan C.**, Qi J., Seregina T., Link C. Jr. Sodium iodide symporter/radioactive iodine system has more efficient antitumor effect in three-dimensional spheroids. ***Anticancer Research*** 2003 May-Jun;23(3B):2397-404.

Hagan, C.R., and Rudin, C.M. Mobile Genetic Element Activation and Genotoxic Cancer Therapy: Potential Clinical Implications. ***American Journal of PharmacoGenomics*** 2002 2(1): 25-35.

Brickley, D.R., Mikosz, C.A., **Hagan, C.R.,** and Conzen, S.D. Regulation of serum and glucocorticoid-induced protein kinase (SGK-1) by ubiquitination. ***Journal of Biological Chemistry*** 2002 277(45): 43064-70.

Invited Presentations

Hagan, C.R., Hillard, C.J., Lange, C.A. Signaling Inputs to Progesterone Receptor Action in Breast Cancer Models. FASEB Summer Research Conference: The Physiology of Integrated Nuclear and Extranuclear Steroid Signaling. August 8-13, 2010.

Hagan, C.R., Hillard, C.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. University of Minnesota Masonic Cancer Center Symposium. June 10, 2010.

Abstracts

Hagan, C.R., Hillard, C.J., Faivre, E.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. Jensen Symposium on Nuclear Receptors. October 14-16, 2009.

Hagan, C.R., Hillard, C.J., Faivre, E.J., Lange, C.A. A common docking domain in the progesterone receptor mediates an interaction with MAPK-phosphatase 3. Gordon Research Conference: Hormone Action in Development and Cancer. July 26-31, 2009.

Hagan, C.R., Hillard, C.J., Lange, C.A. Exploring the role of PR/MEK complex formation in Breast Cancer Models. FASEB Summer Research Conference: Extra-Nuclear Steroid Receptors: Integration with Multiple Signaling Pathways. July 27- August 1, 2008.

Hagan, C.R., Rudin, C.M. JNK is required for SINE transcriptional response following DNA damage. 97th Annual Meeting of the American Association for Cancer Research. April 1-5, 2006. Washington, D.C.

Hagan, C.R., Rudin, C.M. DNA-damage activates transcription of Short Interspersed Elements. FASEB Summer Research Conference: Mammalian Mobile Elements. June 4-9, 2005.

Hagan, C.R., Rudin, C.M. DNA-damage activates transcription of Short Interspersed Elements. 96th Annual Meeting of the American Association for Cancer Research. April 16-20, 2005. Anaheim, CA.

Hagan, C.R., Rudin, C.M. Transcriptional activation of Short Interspersed Elements by genotoxic exposure is attenuated by functional p53. 94th Annual Meeting of the American Association for Cancer Research. July 11-14, 2003. Washington, D.C.

Hagan, C.R., Sheffield, R.F., Rudin, C.M. Induction of Genomic Mobility of SINE Retrotransposable Elements by Genotoxic Exposure. 93rd Annual Meeting of the American Association for Cancer Research, April 6-10, 2002, San Francisco, CA.

Hagan, C.R., Heltemes, L.M., Panchal R.G., Guo, J., Link, C.J. Iodine Uptake and Cell Death in Various Cancer Cell Lines Using the Rat and Human Sodium Iodide Symporter Gene. *Molecular Therapy* 2000 May; 1 (5): S165.

Mitrofanova, E.E., **Hagan, C.R.**, Link, C.J. The Effect of ^{131}I on the Growth of Muticellular Tumor Spheroids Expressing the Sodium Iodide Symporter. *Molecular Therapy* 2000 May; 1 (5): S165.

Guo, J., **Hagan, C.R.**, Panchal, R.G., Mitrofanova, E.E., Qi, J., Wang, S., Link, C.J. Efficient Uptake of Radioisotope into Human Prostate Adenocarcinoma after Rat Sodium Iodide Symporter Gene Transfer Using HSV-1 Amplicon Vector. *Molecular Therapy* 2000 May; 1 (5): S237.

Link, C.J., Heltemes, L.M., Panchal, R.G., Guo, J., **Hagan, C.R.** Radioisotope concentrator gene therapy for cancer with the sodium/iodide symporter gene. American Society of Gene Therapy, Second Annual Meeting, Washington, D.C., June 9-12, 1999.

Link, C.J., Panchal, R.G., **Hagan, C.R.** Heltemes, L.M. Gene therapy for cancer with the sodium/iodide symporter gene. Sixth International Conference of Anticancer Research, October 21-25, 1998, Halkidiki, Greece.